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14. ABSTRACT

Pin1 regulates many factors that are relevant to breast cancer, such as c-Jun, c-Myc, cyclin D1, and cyclin E. However the function of Pin1 in a normal cell is still poorly understood. Thus the role of Pin1 in G0/G1 to S-phase progression of the cell cycle was examined in this research project. The most significant finding was that Pin1 appears to be a novel regulator of the Rb signaling pathway.

15. SUBJECT TERMS
Pin1, Rb, cell cycle, breast cancer

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INTRODUCTION

The purpose of the training grant was to identify the potential mechanism(s) by which Pin1, a peptidyl-prolyl *cis-trans* isomerase, influences breast cancer progression. In order to accomplish this objective, we sought to identify novel roles for Pin1 in the G0/G1 to S-phase transition of the cell cycle, which is a process invariably deregulated in human breast cancer. Previous research has shown that Pin1 can differentially regulate oncoproteins known to control G1/S, including c-Jun, c-Myc, cyclin D1, and cyclin E [1-4]. However these studies have not addressed why Pin1 is required in mouse embryonic fibroblasts (MEF) for efficient cell cycle re-entry from quiescence upon mitogenic stimulation [5, 6]. Thus we have further characterized how Pin1 influences cell cycle re-entry in primary MEF. This annual summary will provide an up-to-date report on the progress made to examine the role of Pin1 in regulating the Rb signaling pathway. In addition, we will discuss our ongoing experiments designed to evaluate the role of Pin1 in genomic instability and breast cancer progression.

BODY

Task 1. Evaluate the role of Pin1 in regulating DNA replication:

Previously we had reported difficulties in accomplishing this task. In particular, MEF were found to be difficult to synchronize in a way that would allow pre-replication complex assembly to be analyzed. The differential populations of quiescent cells in Pin1+/+ versus Pin1-/- MEF cultures also proved to be an issue. Therefore we designed an experiment that addressed these two concerns and evaluated if Pin1 played a role in DNA replication. The rate of [3H]-Thymidine incorporation into exponentially growing Pin1+/+ and Pin1-/- MEF was measured by scintillation counting and normalized by the total number of BrdU incorporating cells as measured by flow cytometry (Figure 1). There was no discernable difference between Pin1+/+ and Pin1-/- MEF in their ability to incorporate [3H]-Thymidine, thus Pin1 does not appear to regulate normal DNA replication.

Task 2. Evaluate the role of Pin1 in regulating p27 during G0/G1-S progression:

Previously we had reported preliminary findings that suggested Pin1 was a negative regulator of p130 (an Rb family member). However the study utilized human WI38 cells transduced with Pin1 shRNA, which showed no cell cycle re-entry defects. Since Pin1-/- MEF have been shown to exhibit a cell cycle re-entry delay, we utilized these cells and more carefully characterized the phenotype using BrdU and Ki67 analysis (Figure 2). In Figure 2A, Pin1-/- MEF show a slight one hour delay in their re-entry into S-phase by BrdU staining. Importantly, the length of S-phase in Pin1-/- MEF (~10.5hr) is nearly identical to Pin1+/+, which is consistent with the findings in Task 1. Cell cycle re-entry in Pin1-/- MEF was also examined by Ki67 staining (Figure 2B), which showed a nearly 2 hour delay and smaller percentage of the cells that released from G0. These data led us to hypothesize that the cell cycle re-entry defect occurred prior to S-phase entry.

In order to find a molecular mechanism for this poorly understood cell cycle re-entry defect, members of MAPK and Rb signaling pathways were analyzed for activation status in response to growth factor re-stimulation. The results showed that the Raf/MEK/ERK signaling cascade was fully functional in the absence of Pin1 (see Appendices). On the other hand, our recent data has shown the Rb signaling pathway to be disrupted in Pin1-/- MEF compared to Pin1+/+ MEF (Figure 3). In particular, the rate of cyclin D1 induction is slower in Pin1-/- MEF compared to wildtype (Figure 3A). Interestingly, Pin1-/- MEF also have lower levels of phosphorylated Rb (Figure 3B). Further research is necessary to determine if Pin1 directly

regulates Rb phosphorylation or indirectly affects phospho-Rb through cyclin D1 regulation. There is some data to support the latter case, however the former has not been studied and would be a novel mechanism for the control of Rb function.

Task 3. Examine the role of Pin1 in human breast cancer:

In order to investigate the role of Pin1 in human breast cancer, we are still currently developing several human primary cell lines using HMEC, BJ cells and WI38 cells, which will be used in soft agar assays and *in vivo* tumorigenesis models. These lines will stably overexpress Pin1 mRNA or Pin1 shRNA along with differing combinations of oncogenes (TERT, p53DD, cyclin D1, cdk4^{R24C}, c-Myc^{T58A} and/or Ras^{G12V}) known to be sufficient to transform human cells [7, 8]. This new six gene model of transformation is particularly useful for this study, since p53, cyclin D1 and c-Myc are all Pin1 targets. These studies will clarify some of the controversy over Pin1's role in cancer, i.e. a tumor promoter or a tumor suppressor, reviewed in [9]. We hypothesize that Pin1 will have differential roles in cellular transformation depending on genetic background and tissue specific differences.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of Task 1. Pin1 does not regulate normal DNA replication.
- Identification of Pin1 as a novel regulator of the Rb signaling pathway.

REPORTABLE OUTCOMES

Poster Abstracts:

Lew, B.O., and A.R. Means (Sept. 2007). Loss of Pin1 does not impinge upon the Raf/MEK/ERK signaling cascade. Duke University Medical Center, Department of Pharmacology and Cancer Biology Annual Retreat, NC. (see Appendices)

Lew, B.O., and A.R. Means (June 2008). Loss of Pin1 accelerates genomic instability in mouse embryonic fibroblasts. Department of Defense, Breast Cancer Research Program, Era of Hope Meeting, Baltimore, MD. (see Appendices)

CONCLUSION

The most significant finding of this ongoing research project is the discovery of a novel regulator of the Rb signaling pathway. Notably, our preliminary data show that Pin1 interacts with p130, Rb and p107 in a cell cycle-dependent manner (data not shown). In addition, the levels of phospho-Rb, p130 and p107 are changed in the absence of Pin1 by a yet unidentified mechanism. Thus a more thorough analysis of the Rb pathway in Pin1-/- MEF is required. Elucidating how Pin1 may regulate the Rb pathway will lead to a better understanding of Pin1's role in promoting cell cycle re-entry from quiescence. In this context, Pin1 would appear to serve a tumor promoting role, which agrees with research done by other groups [10].

Although it seems like Pin1 may be an attractive drug target for breast cancer treatment, there is still no evidence to suggest a causal role for Pin1 in human breast cancer. Our future experiments, including those described in Task 3, will provide insight into how Pin1 might be used as a drug target or a diagnostic tool.

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APPENDICES

Poster Abstracts:

Loss of Pin1 does not impinge upon the Raf/MEK/ERK signaling cascade.

Brian O. Lew and Anthony R. Means

Growth factors can stimulate quiescent mouse embryonic fibroblasts (MEF) to re-enter the cell cycle via the activation of the Raf/MEK/ERK or MAPK signaling cascade. ERK activates cell cycle regulatory proteins, such as p90RSK and c-Myc, by phosphorylating them on serine or threonine residues followed by proline. These pS-P or pT-P motifs can be specifically recognized by the peptidyl-prolyl isomerase Pin1. Previous research has shown the importance of Pin1 in modulating the stability, localization and activity of various substrates, including those involved in cell cycle progression, like c-Myc, c-Jun, cyclin D1 and cyclin E. Interestingly the genetic deletion of Pin1 from MEF results in the impaired ability of quiescent cells to enter S-phase. Hence Pin1-/- MEF were analyzed to determine if MAPK signaling in response to mitogenic stimulation was defective in these cells. Contrary to another study, no defects in MAPK signaling were observed in Pin1 null MEF. However the steady state levels of c-Jun were markedly decreased in the absence of Pin1, which suggests that Pin1 may be involved in immediate early gene expression.

Loss of Pin1 accelerates genomic instability in mouse embryonic fibroblasts.

Brian O. Lew and Anthony R. Means

Background and Objectives:

Pin1 is a conserved peptidyl-prolyl isomerase that specifically recognizes phosphorylated serine/threonine followed by proline motifs (pS/T-P). By catalyzing the cis-trans isomerization of pS/T-P bonds, Pin1 can promote conformational changes in its target phosphoproteins and thereby influence protein stability and/or function. Pin1 has been shown to regulate several proteins that are important in cell cycle progression and breast cancer, including c-Myc, c-Jun, cyclin D1, cyclin E and p53. Interestingly the levels of Pin1 have been found to be upregulated in a number of breast tumor specimens, which has prompted further research on the role of Pin1 in breast cancer. For example, Pin1 ablation from mice on a mixed genetic background was shown to protect them against Ras- or Neu-induced mammary tumorigenesis. Although these studies suggest a tumor promoting role for Pin1 in breast cancer, additional evidence suggests Pin1 can act as a conditional tumor suppressor. Specifically, mouse embryonic fibroblasts (MEF) prepared from Pin1-/- mice on a C57BL6 isogenic background are sensitized to Ras-induced transformation. Though differences in genetic background provide a plausible explanation for these contradictory findings, further investigation is required to elucidate the precise role for Pin1 in breast cancer.

In order to determine how Pin1 is involved in breast cancer, a more thorough understanding of Pin1's function in non-transformed cells is important. Previous research using Pin1-/- C57BL6 MEF led to the finding that Pin1 is a negative regulator of c-Myc and cyclin E. Since the deregulation of these oncoproteins is known to correlate with genomic instability and affect cell cycle progression, the objective of this study was to assess the role of Pin1 in these processes.

Methodologies:

Genomic instability in Pin1+/+ and Pin1-/- MEF was evaluated by quantifying the percentage of micronucleated DAPI-stained nuclei under fluorescence microscopy. Cell cycle progression and aneuploidy were assessed by the flow cytometric analysis of ethanol-fixed and propidium iodide (PI) stained cells.

Results and Conclusions:

The loss of Pin1 from primary and p53-dominant negative (p53DD) expressing MEF led to an increased number of cells with micronuclei. Furthermore Pin1 deletion accelerated the induction of aneuploidy associated with the overexpression of p53DD. These findings suggest Pin1 plays a role in maintaining the integrity of the genome, perhaps through its ability to regulate c-Myc and cyclin E. Interestingly, cell cycle analysis showed that primary Pin1-/- MEF stall in G1 and S-phase, which is consistent with previous research showing the deregulation of cyclin E leads to impaired DNA replication. This study provides evidence that Pin1 can function as a conditional tumor suppressor. Since Pin1 seems to have this dual nature in terms of its role in cancer, perhaps Pin1 could prove to be a useful therapeutic target in breast cancer, but only in the appropriate genetic context.

SUPPORTING DATA

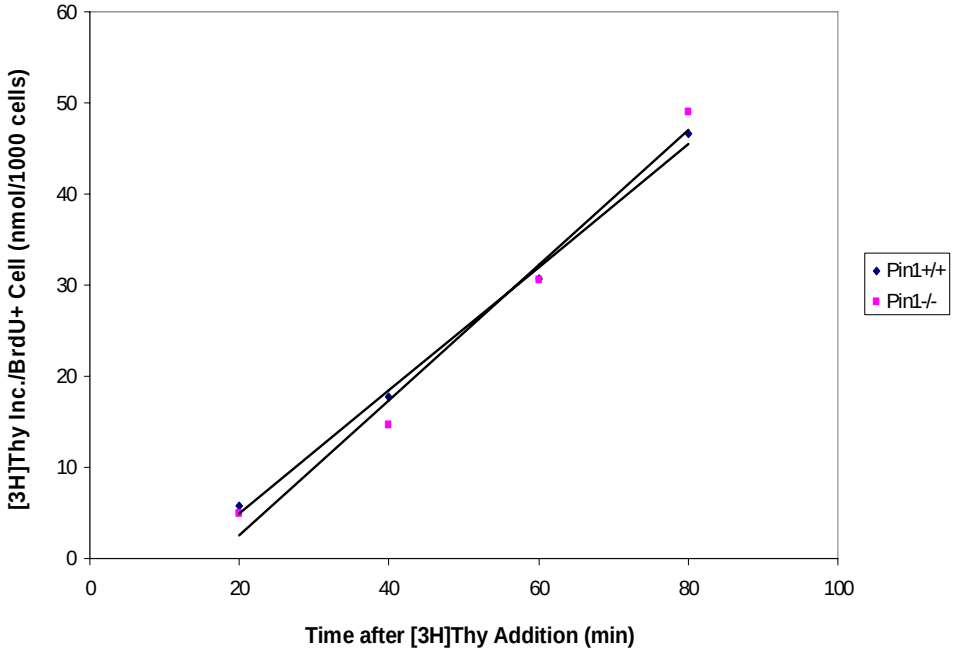

Figure 1. Loss of Pin1 does not affect the rate of DNA synthesis in primary MEF. [3H]Thymidine was added to exponentially growing Pin1+/+ and Pin1-/- MEF. The cells were harvested at the indicated times and analyzed for [3H]Thymidine incorporation by scintillation counting. Data were normalized by the total number of BrdU incorporating cells as determined by flow cytometry.

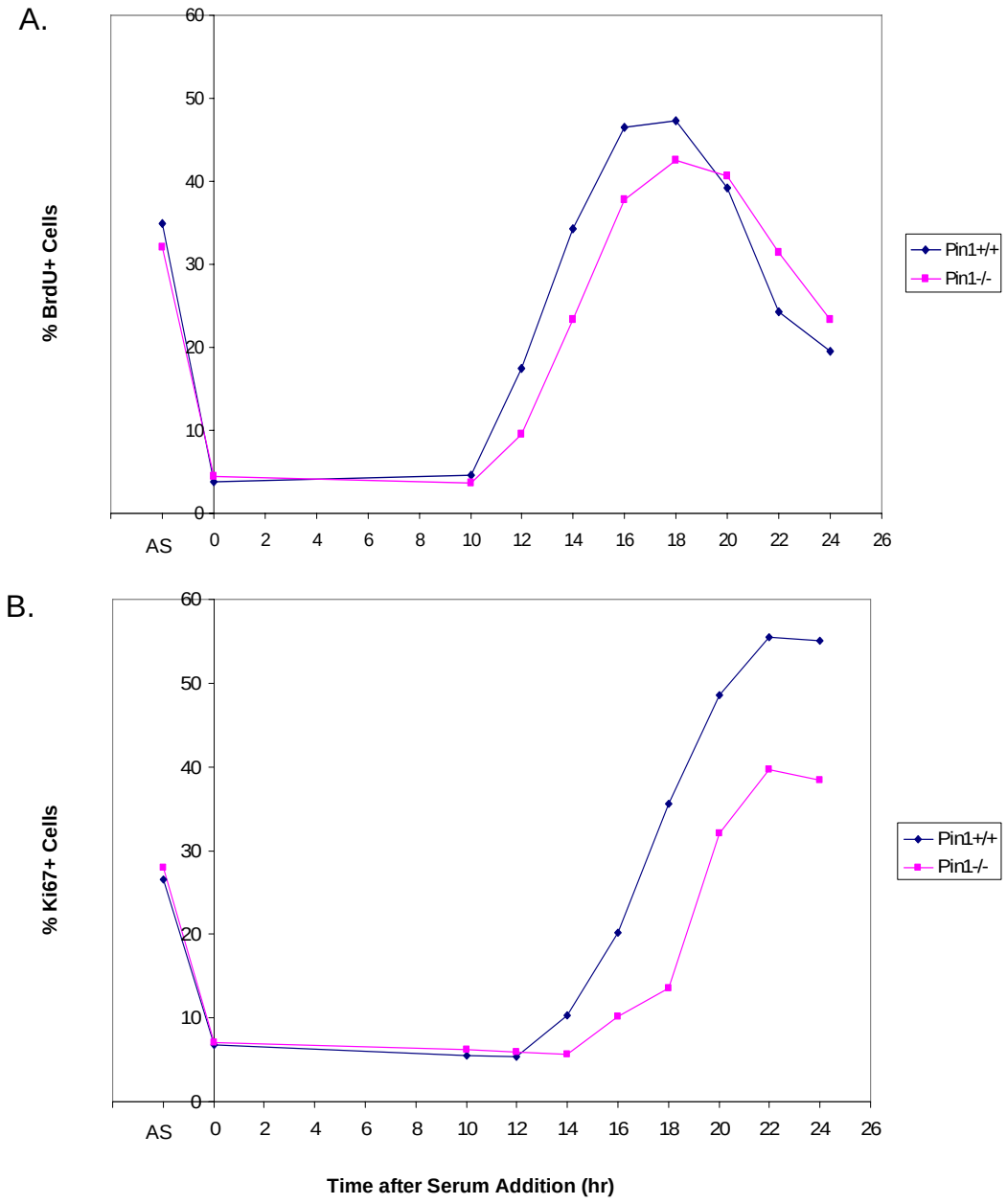

Figure 2. Loss of Pin1 leads to impaired cell cycle re-entry in primary MEF. Asynchronously (AS) growing Pin1+/+ and Pin1-/- MEF were serum starved for 48h. The cells were re-stimulated with 10% FBS and harvested at the indicated times for either (A) pulse-labeling with BrdU or (B) analysis of Ki67 expression by flow cytometry.

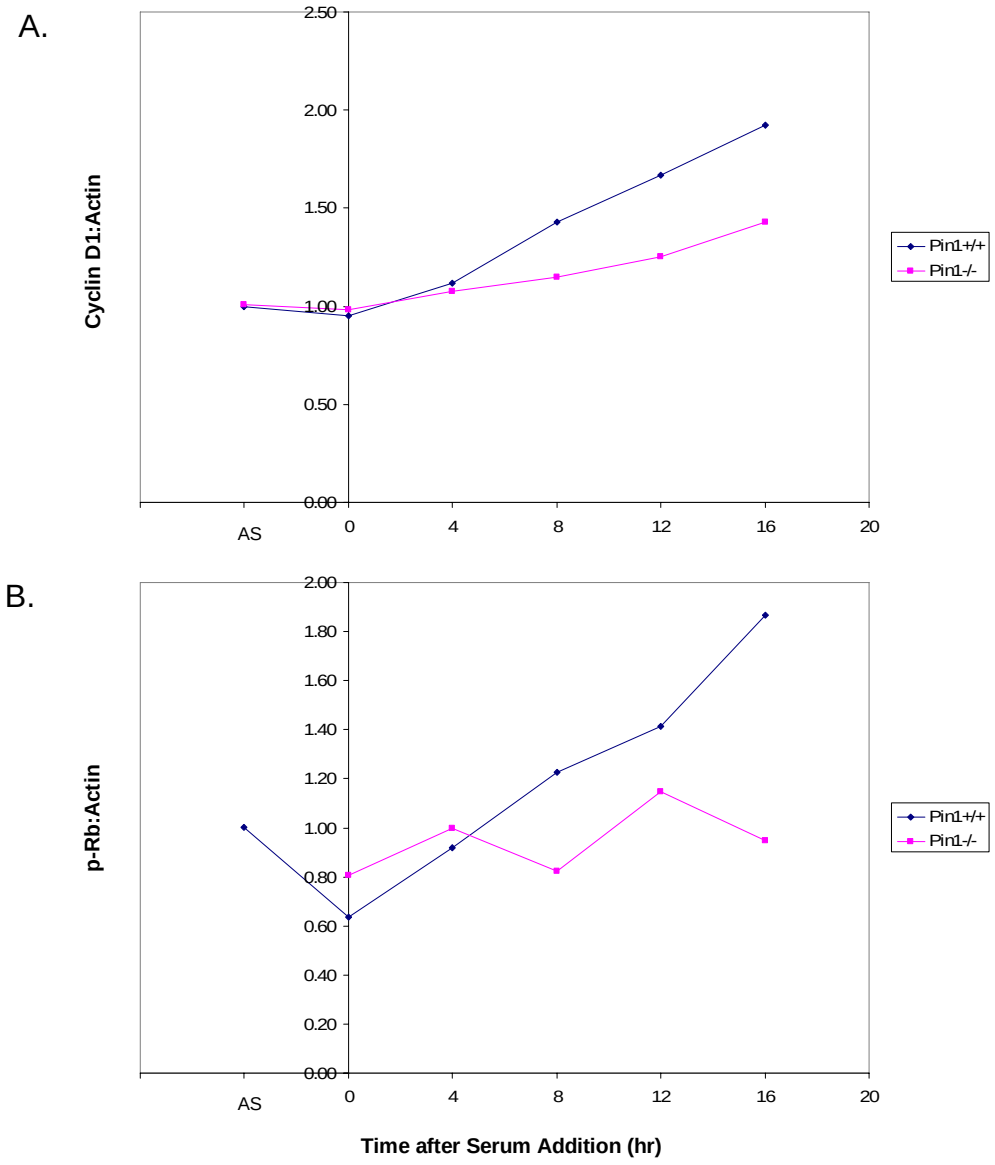

Figure 3. Loss of Pin1 leads to defects in the Rb signaling pathway of primary MEF. Asynchronously (AS) growing Pin1+/+ and Pin1-/- MEF were serum starved for 48h. The cells were re-stimulated with 10% FBS and harvested at the indicated times for western blot analysis of either (A) cyclin D1 or (B) phospho-Rb (S807/S811) levels. Values were determined by densitometry and normalized by actin levels.